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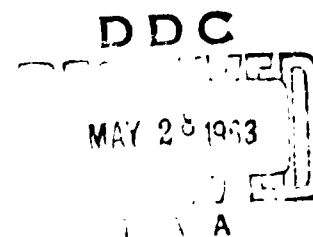
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**ON THE DEMONSTRATION OF  
BACTERICIDAL SUBSTANCES  
IN  
COMMONLY USED CULTURE MEDIA**

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ON THE DEMONSTRATION OF BACTERICIDAL SUBSTANCES  
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Following is the translation of an article by A. Grumbach  
in the German-language periodical *Pathologia et Micro-*  
*biologia* (Pathology and Microbiology) Vol 25, 1962, pp 507-  
516.

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Medical Microbiology of the University of Zurich with the assistance of  
Elisabeth Matthys and Irmgard Lenke.

In a work published in 1902 on bacterial antagonism Lode mentioned  
irritation phenomena appearing at the periphery of containment areas,  
regarding this as a further example of the Arndt-Schulz law, whereby even  
the best nutritive substances, such as peptone, comparable to antidotes  
may inhibit further growth in concentrated solutions.

Subsequently, although not inspired by this comparison--Lode him-  
self fails to substantiate this--numerous articles have been published  
on the germ-damaging culture medium contained substances, continuing to  
be published even now. They not only permitted the exact determination  
of the concept of the "exacting germ", based on empirical consideration  
a representation which could be replaced frequently by the epitheton  
"susceptible", as shown below, especially for these culture media, but  
they also provided greater understanding of the much discussed negative  
phase lag.

Besredka and Jupille showed in 1914 that pneumococci, meningo-  
cocci, gonococci and tubercular bacteria will grow in agar cultures  
only if these are covered with a small quantity of serum (1 to 2 ml per  
Roux cup) or egg bouillon one hour before being vaccinated, "so as to  
impregnate the gelatinous substance with nutritive substances". This  
provided the basis for the concept of a metabolic function of the  
various additive substances.

Drea reached a different conclusion. Based on a report by Corper  
and Uyei he examined in 1940 the effect of the agar, previously con-  
sidered inert, on the growth of a culture assimilated Tb strain (H 37)  
in Long's synthetic culture. He found that even an addition of 0.1 to  
0.25% caused such a strong inhibition that the strain was able to  
develop only after using  $10^{-4}$  mg, while egg cultures have a requirement  
of vaccination of only  $10^{-5}$  to  $10^{-6}$  mg. Based on the agar analysis he  
first thought of Pb intoxication and later of substances which are  
liberated during the greasing, from non-scoured cotton wads or glass  
material and chemically impure culture media, and which are neutralized  
by the addition of serum and massive seeding.

In 1942 Nassau, based on Glass and Kenneth's observations, determined that the addition of blood charcoal to solid (Petragnani) and liquid (Kirchner) cultures permits the growth of even the smallest seeding quantities, and explained it as the absorption of acid metabolic products.

Experiments conducted in 1947 by Dubos and Dubos and assistants followed the same line. Proving that oleic acid in concentrations of 0.000001-0.00001 is able to delay or even inhibit the growth of tubercular bacteria, he furnished for the first time quantitative data on the natural anti-bacterial substances. The inactivation of these and other fatty acids, certain heavy metals, chloric and phenolic compounds, anionic and cathionic and non-ionized wetting agents, the latter having been synthesized by Dubos and designated tweens, was successfully accomplished by means of serum albumin.

Regardless of whether long-chained fatty acids detoxicated by esterification, serum albumin or blood charcoal have a growth-promoting function--according to Hirsch (1954a) they can replace any other C source--visible growth was observed following the feasibility of seeding synthetic cultures containing liquid, detoxicated tweens with only  $10^{-8}$  mg of bacteria after a few days. This destroyed the thesis of the metabolic function of massive seeding and the addition of egg yolk, serum, plasma, etc. in favor of its detoxicated effect. However, the question remained open as to the nature of the substances which were effective in isolated cases.

The question of metabolism or a neutralizing factor also arose regarding the culture of meningococci and gonococci, which only grew following the addition of blood, serum, ascites or egg yolk in the usual meat broth pepto cultures. In 1916 Lloyd was able to replace animal protein with pea powder or starch, based on Gordon's theory. According to her era she presumed that these additives were the carriers of the vitamins essential to the Neis series, an opinion which Mieschner rejected as being inadequate for their requirements. Glass and Kenneth also considered charcoal, especially blood charcoal, which promoted the growth of these Neis series on agar plates in a  $CO_2$  atmosphere primarily as a metabolic effect. Based on Warburg's work they thought of a catalytic oxydation of oxalic acid and the possibility of the absorption of a toxic substance. This viewpoint was adopted by Wyon and McLeod in 1923. McLeod, together with Gordon and later on with Wheatley and Phenon, considered these to be excess amino acids caused by peptone.

Mueller's and Hinton's experiments went along the same lines, showing that both Neis series can be grown in a single culture so long as the growth inhibiting factors attached to them are removed by means of a starch.

In 1947 Lwoff, based on the above experimental results of Dubos (1947) with mycobacteria, adopted the thesis of the growth-inhibiting effect of "any toxic substances found in the common meat broth pepto cultures." He also saw "that gonococci occasionally grew without the addition of serum and that the bouillon became useful following the

addition of small quantities of (1/100,000) Na cholesterine sulphate or by dilution with distilled water or 0.6% NaCl solution, and showed that peptone extracted for 48 hours in Soxhlet with ether was less toxic than the original material.

Analogously Ley and Mueller extracted the toxic substance suspected by Drea to be contained in the agar with methanol--all other organic solutions were ineffective--and concentrated this material by means of ether extraction such that 0.5  $\mu$ g/ml inhibited the growth of the gonococci and became inactivated in this fashion by means of starch. From the fact that butterfat acids extracted by means of alkaline hydrolysis (stearic and oleic acids) behaved like quantitatively equal inhibitors they deduced that there was a material similar to fatty acids.

Frantz's first success with meningococci cultures in liquid synthetic cultures--agar proved to have an inhibiting effect--proved that these germs are not the exacting kind, but the kind sensitive to certain amino acids, fatty acids and other toxic substances (Gerhardt and Heden).

In 1936 Garrod pointed out that staphylococci, which usually grow in agar containing meat broth peptone cultures do not take with a small inoculation in the common bouillon. Since the addition of 40% of a Berkefeld filtrate of a 24-hour culture of staphylococci also enabled a small number of germs to grow, and the filtrate of a 48 hour culture being even better, he concluded that there was a metabolic effect formed by the massive seeding of direct bacteria, and believed that this was in agreement with an earlier observation by Churchman and Kahn, according to which a *E coli* grew less the greater the bouillon volume.

In contrast O'Meara and Macsween confirmed Dubos' 1929 opinion on the effect of redox potentials being "one of the numerous factors affecting growth", and in addition verified that the culture acts not only as a bacteriostatic, but also as a bactericide. They considered the carrier of the substance inactivated by boiling to be copper, occasionally found in large quantities in the peptone, which was inactivated completely by the serum during a streptococci experiment.

In 1959 Meyer reported the existence of natural water with a deadening effect on typhus and paratyphus bacteria (death rate Q - seeding through harvesting - greater than 1) and was able to attribute this to a sometimes stronger sometimes weaker lethal factor, subject to absorption by  $\text{Fe}(\text{OH})_3$  and extracted from a physiological NaCl solution of a thermal stable nature, which, however, could not be isolated by means of the orifice test on agar plates.

#### Our own experiments

Systematic experiments with bacterial antibiotics led us to the initially reported questions. Even though, based on the results obtained with massive seeding, it could not be expected that the toxic substances characteristic of the culture would interfere in all cases with those formed by the bacteria, the experiment to remove the often suspected and empirically inactivated substance at least in part from the culture and to test its effect seemed worthwhile.

After many trials we used ether for the extraction, and later only acetic acid ethyl ester. To extract iron hydroxide we used the method indicated by Meyer (1959a).

#### Extraction methods

##### a) Acetic acid ethyl ester extraction:

One liter of bouillon is extracted twice with 250 ml of acetic acid ethyl ester each. After separation in the separating funnel the extract is mixed with some  $\text{Na}_2\text{SO}_4$  to remove the water, left standing at room temperature until clear, then filtered and compressed under vacuum at 50°C to 0.5 ml. The concentrate was always dissolved in 10 ml physiological NaCl solution in order to obtain comparable values. In this form it has a pH factor of about 4 and keeps for months at +4°C.

##### b) Protein hydroxide extraction:

100 ml bouillon are first mixed with 2 ml  $\text{FeCl}_3$  (514.35 mg%) and then with 2 ml n/10 NaOH and left standing until flocculation is complete at room temperature. Contrary to Meyer (1959a) we used a hard filter (LS 16 1/2), eluted the precipitate in physiological NaCl for two days at room temperature and compressed the liquid in a water bath at 70°C to 10 ml.

#### Experimental layout

The extracts were tested in the cylinder test--0.2 ml porcelain cylinder with 8 mm O.D. and in the strip test--5 mm wide and 80 mm long with filter paper strips soaked in 0.1 ml extract. The 18-hour old germs were inoculated in liquefied meat broth peptone agar and this was molded into plates. For the pneumococci and neis series we used 10% serum agar; the latter were superficially smoothed out with a spatula and incubated in a 10%  $\text{CO}_2$  atmosphere.

#### Experimental results

The figures contained in Tables I through III represent diameters of the inhibition areas in millimeters obtained with the acetic acid ethyl ester extracts.

Table I

Cylinder test with acetic acid ethyl ester extracts from sterile uncovered 1% peptone containing meat broth bouillon.

Test germs		Test germs	
<i>B. anthracis</i>	26	<i>K. pneumoniae</i>	12
staphyloc. penic. res.	24	<i>E. coli</i> O 26	13
staphyloc. penic. sens.	17	<i>E. coli</i> O 111	14
sc. haemolyt. A	16	<i>Ps. pyocyanea</i>	Spur
sc. haemolyt. B	14	<i>S. typhi</i> H 901	25
sc. haemolyt. C	17	<i>S. typhi</i> Vi	18

[Table continued, next page]

<i>Sc. haemolyt. D</i>	13	<i>S. typhi murium</i>	17
<i>B. megatherium</i>	20	<i>S. enteritidis</i>	19
<i>G. tetragena (Basel)</i>	34	<i>Sh. sonnei</i>	16
<i>N. gonorrhoeae</i>	23	<i>Sh. large-Sachs</i>	21
<i>N. meningitidis</i>	24	<i>Proteus H</i>	20
<i>D. pneumoniae Typ III</i>	22	<i>Proteus OX 19</i>	22

Table I shows the values obtained on 12 gram positive and 11 gram negative germs from meat broth peptone bouillon extracts.

These are not absolutely comparable among themselves because the germ density of the fluid inoculated agar tubes could be kept only vaguely constant. At any rate, it is noteworthy that during repeated experiments only *Ps. pyocyanea* was almost insensitive to the extract.

Tables II and III show the results obtained for extracts obtained from 13 different bouillons.

In the cylinder test (Table II) we tested for *B. anthracis*, *K. pneumoniae*, *N. meningitidis* and *N. gonorrhoeae*. Here too the figures are not absolutely comparable, due to possible differences in germ densities. At any rate they reflect the fact that *B. anthracis* and *N. gonorrhoeae* are basically more sensitive than *Kl. pneumoniae* and *N. meningitidis*, while the peptone content is of no substantial consequence.

Table II  
in common culture media

Cylinder test with acetic acid ethyl ester extracts from sterile, uncovered culture media

Culture media	Test germs			
	B anthracis	Kl pneumoniae	N 4939 meningitidis	N 70 gonorrhoeae
Meat br. bouill. w/o pep.	41	21	31	51
Meat br. bouill. with pep.	36	16	26	42
dextr. meat br. b. w/o pep.	28	15	23	33
dextr. meat br. b. with pep.	31	17	23	35
Liebig bouillon w/o peptone	32	13	24	33
Lieb. bouill. with peptone	41	21	23	38
Lieb. bouill. w/dextr w/o pep	40	18	23	26
Lieb. bouill. w/dextr. with pep	31	19	21	26
DIFCO dextrose broth	36	20	28	18
DIFCO heart infusion	33	16	23	18

[Table continued, next page]

Vitambact bouillon	36	18	21	20
Peptone water with 1% dex, 1% serum	35	13	20	23
Yeast malt extract bouillon	40	21	56	18

Table III

5mm filter paper strip test with 0.1 mm acetic acid ethyl ester  
extracts soaked in uncovered culture media

Cultures	spleen br.	gland	staph	Test germs		Coli	Ps pyoc	S enter
				strep A	str D			
Meat extract bouillon w/o peptone	18	8	10	13	-	-	-	-
Meat extract bouillon with peptone	14	8	8	8	-	-	-	-
dextrose bouillon w/o peptone	13	8	8	-	-	-	-	-
dextrose bouillon with peptone	13	10	10	-	-	-	-	-
Liebig bouillon w/o peptone	12	10	10	-	-	-	-	-
Liebig bouillon with peptone	17	10	10	-	-	-	-	-
Lieb. bouill. w/dextr. w/o peptone	15	11	10	10	-	-	-	-
Lieb. bouill. w/dextr. w/peptone	15	10	10	8	-	-	-	-
DIFCO dextrose broth	17	10	10	-	-	-	-	-
DIFCO heart infusion	22	8	8	10	-	-	-	-
Vitambact bouillon	12	-	-	-	-	-	-	-
Peptone water w/1% dext, 1% serum	15	-	-	9	-	-	-	-
10% serum in dist. water	12	-	12	-	-	-	-	-
Yeast malt extract bouillon	29	21	7	-	-	7	21	-
Glycocol buffer pH 3.0	-	-	-	-	-	-	-	-

That the inhibit zones registered in Table III, determined with the paper strip test and corresponding to the much lower extract concentration (0.1 ml/40 mm<sup>2</sup> against 0.2 ml/20mm<sup>2</sup> in the cylinder test) are smaller, and that the spleen bacilli proved to be the more sensitive germs in this experiment was to be expected. However, it was surprising that besides Ps. pyocyanea the enterococci strain and the E. coli and S. enteritidis failed to react at all. This unusual effect of the bactericidal extract leads to the suspicion that it is caused by various factors.

We speak about bactericide since we failed in many experiments to induce any resistance or to isolate resistant mutations. The germs contained in the inhibit areas always lost their gram positivity very rapidly, and after a few hours could no longer be over inoculated.

That in spite of the acid nature of the extracts and in spite their loss of activity due to neutralization no pH effect is involved is substantiated by the numerous, always negative controls with the corresponding glyccocol buffer solutions.

Confirming Meyer's findings (1959a) no growth inhibitions were ever encountered during the orifice test with  $\text{Fe}(\text{OH})_3$  extracts.

#### Summary

The toxic substances which have repeatedly been suggested to be present in the commonly used meat infusion peptone broth can be extracted at least partially from various nutrient media with acetic acid ethyl ester and their action tested by cylinder and paper strip method. Their action is bactericidal and selective. Anthrax bacilli were consistently most susceptible, while *Pseudomonas aeruginosa* was almost resistant.

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